Validation of the veterinary diagnostic product "*FAST*est® NEOSPORA caninum **test-kit"**

MEGACOR Diagnostik GmbH has developed a rapid test for the detection of anti-*Neospora caninum* antibodies in whole blood, serum or plasma of dogs and cattle. The test is based on an immunochromatographic reaction, where *Neospora caninum a*ntibodies present in the sample are bound to membrane fixed, recombinant *Neospora caninum* antigens. The interpretation is qualitative. A control line appears confirming the test functionality in principle. In case of a positive sample, an additional *Neospora caninum*-specific test line appears on the test membrane.

The Institute of Parasitology of Vetsuisse Faculty Bern, Länggass-Str. 122, 3001 Bern was authorized by MEGACOR to run a sensitivity testing of the MEGACOR veterinary test named above.

The study contains following issues:
1. Check-up of the provided ***FAST*est® NEOSPORA** caninum test-kits
2. Results of sensitivity testing
3. Summary and discussion

1. Check-up of the provided *FAST*est® NEOSPORA caninum **test-kits**

1.1 Test packaging and labelling
The labels of the test-kit contain following information: test name, summary about all test components (bilingual, German and English) with quantities, batch No, expiry date and storage instructions. All test-kit components named in the summary are proved. All provided test-kits used in the study were of the same batch 1803 with expiry date 31.12.2015.

1.2. Test procedure
The "Instructions for use" is available in German and English version and classified in following chapters:

1) Information on the test-kit including test-kit components
2) Introduction
3) Information on the specimen material
4) Specimen collection and preparation
5) Test procedure
6) Reading of the test result
7) Precautions for users
8) Test principle
9) Information for the interpretation

The technical instruction is detailed and understandably written. All necessary data for required volumes, necessary amount, temperatures and time specifications for test procedure are included. Possible errors which could influence the test result are pointed in detail. Special comments are listed in the chapter Precautions for users. Information for test interpretation is comprehensive.

2. Study procedure

In the reference lab, positive and negative sera from dogs and cattle were examined by ***FAST*est® NEOSPORA** caninum test-kit**.** Additionally, sera from cattle experimentally infected with *Neospora caninum* were tested.
All sera were tested with IFAT to define their positive or negative status. Sera > 1:160 in IFAT (cut off) are to be interpreted positive. Sera with titre of 1:160 and weak fluorescence were defined as doubtful.

The relative sensitivity was defined using *Neospora caninum* positive sera with IFAT cut off ≥ 1:160. The relative specificity was defined using *Neospora caninum* negative sera with IFAT cut off < 1:160. Potential cross-reactions were checked with *Babesia canis*, *Leishmania* sp. and *Toxoplasma* sp. positive dog sera as well as with *Babesia divergens* and *Toxoplasma* sp. positive bovine serum samples. Test results were read after 15 minutes as required in the instructions for use and additionally after 20 and after 30 minutes.

3. Results and Interpretation

In total, 18 test-kits (180 tests) of the same lot were provided by MEGACOR Diagnostik GmbH. The intensity of the control lines vary from weak to strong. The reaction patterns of the test lines vary from sharp and distinct to broad and vaguely dispersed. As written in the instructions for use of positive T-line reactions are represented by any pink/purple coloured line appearing at the appropriate position after 15 minutes incubation time. Sera defined negative do not show any T-line reaction at all. T-lines which were extremely difficult to see and which could not be interpreted clearly were defined neither positive nor negative. From these, only the reaction pattern/colour was described. Due to manufacturer, sera with very low antibody titre could show a gradation of reaction due to reaction time and T-line intensity. This could only be partially proven in our study. After the official reading time of 15 minutes incubation time, some sera showed altered T-line patterns. Therefore, an increasing amount of unspecific reactions was stated after 20 minutes incubation. At the additional reading time of 30 minutes no further changes were seen.

3.1 Results of dog sera

All 18 negative defined sera (golden standard = IFAT) also show negative test results in the ***FAST*est® NEOSPORA** caninum. None of the sera shows an unspecific reaction (see table 1).

In total, 7 sera with low antibody IFAT titres (1:40, 1:80) and one serum with IFAT cut off titre (1:160 1 +/-) also were tested with ***FAST*est® NEOSPORA** caninum. 5 of them show a negative ***FAST*est® NEOSPORA** caninum test result, 3 (including the 1:160 +/- serum) show a weak test line that can be interpreted only with difficulty after 15-20 minutes (see table 2).

All positive defined dog sera show a positive result with a clearly defined test line in ***FAST*est® NEOSPORA** caninum**.** A gradation of reaction (T-line intensity) is seen in some serum samples (see table 3).

Tests for cross reactivity (see table 4) show in only 1 of 5 defined *Toxoplasma* sp. positive sera an unspecific reaction using ***FAST*est® NEOSPORA** caninum. Also, serum of a double-infected (*Leishmania* and *B. canis*) dog reacted with an unspecific T-line. Sera from monoinfected (*Leishmania* or *B. canis*) dogs showed no cross reaction.

3.2 Results of bovine sera

The testing of bovine sera showed comparable results. All negative defined sera (golden standard = IFAT) also show negative test results in the ***FAST*est® NEOSPORA** caninum (see table 5). The high specificity could be also confirmed using the bovine sera.

The investigation of sera with low titres and sera with borderline values (1:80, 1:160 1 +) showed 1 positive (serum 13S407) and in 6 of 9 sera several unspecific and partially very difficult interpretable reactions (see table 6) using ***FAST*est® NEOSPORA** caninum. The positive reacting bovine serum showed a titre of 1:80 using IFAT. For us, this sample was presumably from a latent infected cattle and therefore we stated no false positive result.

From 39 positive IFAT defined bovine sera 37 show a positive test result and 2 a negative test result using the ***FAST*est® NEOSPORA** caninum (see table 7). As seen in the dog sera, some sera showed a gradation of reaction (T-line intensity). There was no cross reactivity testing for bovine positive *Toxoplasma* sp. and *B. divergens* sera with ***FAST*est® NEOSPORA** caninum (see table 8).

3.3 Sensitivity and Specificity

For the evaluation of the relative sensitivity, in IFAT positive defined *Neospora caninum* sera (≥ 1:160) were used. From total 52 IFAT positive defined canine and bovine samples 50 samples reacted positive using ***FAST*est® NEOSPORA** caninum. Result is a relative sensitivity of 96.2%.

For the evaluation of the relative specificity, in IFAT negative defined *Neospora caninum* sera (<1:160) were used. All 37 IFAT negative defined canine and bovine samples reacted neagtive using ***FAST*est® NEOSPORA** caninum. Result is a relative specificity of 100%.

4. Summary and reference

***FAST*est® NEOSPORA** caninum test-kit is clearly designed. Test procedure is easy and time saving.
The test results are qualitative. A quantification of results is not possible.
Reading of the test result should be strictly after 15 minutes, due to increasing unspecific reactions between 15 and 20 minutes, respectively, and increase of results that are more difficult to interpret.

In view of the reference lab for neosporosis, ***FAST*est® NEOSPORA** caninum test-kit of the company MEGACOR complies to all requirements for a sensitive(relative sensitivity of 96.2%) and specific (relative specificity of 100%) rapid test for the detection of *N. caninum* in dog and cattle. Unspecific reactions should be clarified with a complementary examination (IFAT, ELISA).

Appendix

Table.1: Results of the dog sera tested *Neospora caninum* negative in IFAT

No.	**Sample No.**	**IFAT**	***FAST*est NEOSPORA caninum Test-kit**
1	11S763	Negative	Negative
2	11S885	Negative	Negative
3	11S963	Negative	Negative
4	11S995	Negative	Negative
5	12S163	Negative	Negative
6	12S164	Negative	Negative
7	12S268	Negative	Negative
8	12S357	Negative	Negative
9	12S470	Negative	Negative
10	12S487	Negative	Negative
11	12S507	Negative	Negative
12	13S96	Negative	Negative
13	13S137	Negative	Negative
14	13S178	Negative	Negative
15	13S309	Negative	Negative
16	13S402	Negative	Negative
17	13S410	Negative	Negative
18	13S503	Negative	Negative

Table.2: Results of the dog sera tested marginal and in low titres in *Neospora caninum* IFAT

No.	**Sample No.**	**IFAT**[1)]	***FAST*est NEOSPORA** caninum **Test-kit**
19	085215	1/80 +/-	Negative
20	095130	1/40 +/-	Negative
21	095613	1/40 +/-	Negative
22	10515	1/40 +/-	Negative
23	105749	1/160 +/-	weak band
24	115440	1/80 +/-	weak band
25	12571	1/80 +/-	weak band
26	125321	1/80 +/-	Negative

1) Marginal titre IFAT: 1/160

Table.3: Results of the dog sera tested *Neospora caninum* positive in IFAT

No.	**Sample No.**	**IFAT**	***FAST*est NEOSPORA** caninum **Test-kit**
27	11S1140	1/1280	POSITIVE
28	13S1622	≥ 1/5000	POSITIVE
29	13S274	1/4500	POSITIVE
30	75/95	1/320	POSITIVE
31	227/95	≥ 1/320	POSITIVE
32	1083	≥ 1/320	POSITIVE
33	782	1/160	POSITIVE
34	1232	≥ 1/320	POSITIVE
35	1536	1/160	POSITIVE
36	1010	1/320	POSITIVE
37	1042	1/160	POSITIVE
38	1178	1/160	POSITIVE
39	4363	1/160	POSITIVE

Table.4: Results of testing of dog sera on cross reactions in ***FAST*est NEOSPORA** caninum

No.	**Sample No.**	**IFAT Titre**		**ELISA (AE)1)**	**IDEXX2)**	***FAST*est**
		Toxoplasma sp.	***Babesia canis***	***Leishmania sp.***	***Dirofilaria sp.***	**NEOSPORA** caninum **Test-kit**
40	12S478	≥ 1/160				Negative
41	125507	≥ 1/160				Negative
42	TS9598	≥ 1/320				Negative
43	TS4247	≥ 1/320				Negative
44	1622/664	≥ 1/160				Weak diffuse broad band
45	826/96		≥ 1/1280			Negative
46	1529/844		≥ 1/160	84		Weak diffuse broad band
47	13S753			131		Negative
48	521/279				POSITIVE	Negative

1) ELISA Evaluation Leishmaniose: positive from 1 AE

2) Qualitative test, no titre information possible

Table.5: Results of the cattle sera tested *Neospora caninum* negative in IFAT

No.	**Sample No.**	**IFAT**	***FAST*est NEOSPORA** caninum **Test-kit**
1	135246	Negative	Negative
2	135250	Negative	Negative
3	135254	Negative	Negative
4	135276	Negative	Negative
5	13S277	Negative	Negative
6	13S278	Negative	Negative
7	135279	Negative	Negative
8	135280	Negative	Negative
9	13S281	Negative	Negative
10	135282	Negative	Negative
11	13S283	Negative	Negative
12	135284	Negative	Negative
13	13S334	Negative	Negative
14	135429	Negative	Negative
15	125574	Negative	Negative
16	12S593	Negative	Negative
17	135592	Negative	Negative
18	135593	Negative	Negative
19	13S594	Negative	Negative

Table.6: Results of the cattle sera tested marginal and in low titres in *Neospora caninum* IFAT

No.	**Sample No.**	**IFAT**[1)]	***FAST*est NEOSPORA** caninum **Test-kit**
20	13S175	1/80+/-	Small clear band
21	135255	1/160+/-[2)]	Negative
23	13S387	1/80+/-	Weak band with diffuse zone
24	135406	1/80+/-	Clear band with diffuse zone
25	13S407	1/80 +/-	POSITIVE
26	135408	1/160+/-[2)]	Negative
27	135520	1/80+/-	Clear band with diffuse zone
28	135555	1/80+/-	Negative
29	13S573	1/80+/-	Weak small band

1) Threshold titre IFAT: 1/160
2) Threshold reaction with 1/160

Table.7: Results of the cattle sera tested *Neospora caninum* positive in IFAT

No.	**Sample No.**	**IFAT**[1)]	***FAST*est NEOSPORA** caninum **Test-kit**
30	12S592	≥ 1/160	POSITIVE
31	12S594	≥ 1/640	POSITIVE
32	13S640	≥ 1/160	POSITIVE
33	13S179	≥ 1/160	POSITIVE
34	RD4/52	≥ 1/160	POSITIVE
35	3510	1/320	POSITIVE
36	3514	≥ 1/320	POSITIVE
37	3515	≥ 1/320	POSITIVE
38	3521	1/320	POSITIVE
39	3524	1/160	POSITIVE
40	3537	≥ 1/320	POSITIVE
41	3562	≥ 1/320	POSITIVE
42	3564	≥ 1/320	POSITIVE
43	3571	≥ 1/320	POSITIVE
44	3620	1/640	POSITIVE
45	3621	≥ 1/320	POSITIVE
46	3632	≥ 1/320	POSITIVE
47	3639	1/320	POSITIVE
48	3641	1/320	POSITIVE
49	3642	1/160	POSITIVE
50	3643	1/320	POSITIVE
51	3662	1/320	POSITIVE
52	3678	1/160	POSITIVE
53	3679	≥ 1/320	POSITIVE
54	3683	1/320	POSITIVE
55	3684	1/160	POSITIVE
56	3692	1/320	POSITIVE
57	3690	1/320	POSITIVE
58	3696	1/320	POSITIVE
59	3701	1/160	Negative
60	3714	1/320	POSITIVE
61	3719	1/160	Negative
62	3720	1/160	POSITIVE
63	3975	1/320	POSITIVE
64	3977	≥ 1/320	POSITIVE
65	3988	1/160	POSITIVE
66	4016	1/160	POSITIVE
67	4034	1/160	POSITIVE
68	4140	1/160	POSITIVE

1) Threshold titre IFAT: 1/160

Table.8: Results of testing of cattle sera on cross reactions in ***FAST*est NEOSPORA** caninum

No.	**Sample No.**	**IFAT titre**		***FAST*est NEOSPORA** caninum **Test-kit**
		Toxoplasma sp.	***Babesia divergens***	
69	HPTOX	≥ 1/160		Negative
70	NPTOX	≥ 1/160		Negative
71	RD2/0	≥ 1/160		Negative
72	HPBDIV		≥ 1/160	Negative